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Modulation of longevity in *Daphnia magna* by food quality and simultaneous exposure to dissolved humic substances

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ABSTRACT

Various abiotic and biotic factors determine the natural fluctuations of *Daphnia* spec. populations; food quality and dissolved humic substances (HSs) being among these factors. In this contribution, we try to disentangle the relative impact of food quality and simultaneous HSs exposure on the fertility and longevity of *D. magna*. It is understood that HS-mediated stress leads to reduced fecundity in well-fed *D. magna* females; hence, it was expected that poor food, as a second stressor, would aggravate the HS-mediated effects. Three diets were tested: the green algae *Pseudokirchneriella subcapitata*, baker's yeast alone, and baker's yeast plus dissolved ascorbic acid, and exposed *D. magna* to a HS preparation which has been shown effective in previous bioassays. It was hypothesized that the lifespan and fertility of *D. magna* would be best when fed green algae, and worst when fed only baker's yeast. However, contrary to these expectations, any addition of HSs reduced the stress caused by poor food quality and increased lifespan and fecundity. In the yeast series, asexually produced diapausing eggs occurred via a so far unknown pathway. With yeast diet, the expanded lifespans were slightly above, whereas the increased offspring numbers lay below, the corresponding data of the algae-fed individuals. The potential of HSs as an additional food source and as a means to extend the lifespan is discussed. These findings open the innovative perspective that under low quality food conditions, additional stressors at certain intensities may even be beneficial to individuals and populations.

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Introduction

Many abiotic and biotic factors determine the natural fluctuations of zooplankton population densities in general, and *Daphnia* in particular: climatic parameters, such as temperature and light (Berberovic et al. 1990; Straile and Geller 1998; Alekseev and Lajus 2009), pH, oxygen conditions, and other water quality parameters (Krause-Dellin and Steinberg 1986), quantitative and qualitative food conditions (Geller 1975; Moore 1980; Rohrlack et al. 1999), essential nutritional elements and compounds (e.g., Müller-Navarra 1995; Martin-Creuzburg et al. 2006, 2008), as well as predation by fish or invertebrates (e.g., Vanni 1986; Lampert 1993). Even these factors create a complex tangle of influences (Gyllström and Hansson 2004); yet, the situation becomes even more complex, since one ecological parameter has not yet been considered as a stressor in depth: dissolved organic carbon, DOC (Steinberg et al. 2006, 2008). In non-eutrophicated freshwater systems, the DOC content exceeds the organic carbon in all organisms by approximately one order of magnitude (Wetzel 2001; Steinberg 2003), and its majority is comprised of dissolved humic substances, "HSs", (Thurman 1985). Freshwater organisms are intimately exposed to concentrations between < 0.1 and > 10 mmol l^{-1} HSs (Steinberg et al. 2008).

The presence of various different functional groups enables HSs to interact with organisms even on the subcellular, biochemical and molecular biological level; consequently, they have the potential to act as natural chemical stressors (Steinberg et al. 2003). Internalized HSs lead to the activation of oxygen as one major response pathway (Timofeyev et al. 2006; Meinelt et al. 2008) and induce anti-stress reactions which are energy consuming and may be embryotoxic or even lethal (Steinberg 2003; Cazenave et al. 2006; McMaster and Bond 2008). As with many other stresses, the HS-mediated stress may also be overcome, at least partly, if the diet of the exposed animals contains spontaneous antioxidants, such as ascorbic acid. There are indications that different diets have a significant impact on the antioxidant response of aquatic invertebrates (Elser et al. 2000; Jensen and Hessen 2007; Timofeyev et al. 2009), particularly excess of carbon leads to oxidative stress (Darchambeau et al. 2003).

On the other hand, HSs may serve as an indirect or even direct food and energy source (Gellis and Clarke 1935; Haney 1973;

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Salonen and Hammar 1986; Hessen et al. 1990) and overcome shortages of other food sources. Furthermore, HSs have the potential to expand the lifespan of exposed individuals, as shown so far with the nematode, *Caenorhabditis elegans* Maupas, and the water fleas, *Daphnia magna* Straus (Steinberg et al. 2007; Euent et al. 2008) and *Moina macrocopa* Straus (Suhett et al., unpublished data). With the first water flea species, life extension was gender-specific: the lifespan of males increase, whereas females had a reduced lifespan and fecundity (Euent et al. 2008).

To disentangle and assess the relative impact of two co-occurring environmental factors on the fertility and longevity of *D. magna*, food quality and HSs exposure was tested and it was assumed that HSs may serve as a food and energy source best under poor food conditions. It was presumed that any stressor alone would reduce both the fertility and longevity of *D. magna*. Consequently, and according to the aforementioned findings about HSs as natural chemical stressors, it was hypothesized that a second stressor, such as poor food quality, aggravates the effects of the first one, for instance HSs. Consequently, it was thus expected that the lifespan and fertility of *D. magna* were best when fed *P. subcapitata*, and worst when fed only baker's yeast, and intermediate when fed baker's yeast amended with dissolved ascorbic acid.

Material and methods

D. magna clone

The applied clone of *D. magna* was obtained from the German Federal Environment Agency, at that time in Berlin. *D. magna* was cultured in ADaM artificial *Daphnia* medium (Klüttgen et al. 1994), yet with a 10-times higher bicarbonate content for 5 generations prior to exposure. The increased bicarbonate content was applied to buffer against acidification by ascorbic acid addition in one treatment.

Feeding scenarios

Daphnia magna was fed with three different diets.

(1) The green alga, *Pseudokirchneriella subcapitata* (Koršikov) Hindák, with an average diameter of 3.85 μm (measured by Cell Analyser System, CASY, Schärfesystem, Reutlingen, Deutschland), was used as food, since this species has proven to be a high quality food with several cladoceran species (Muñoz Mejía and Martínez Jerónimo 2007) and rich in ascorbic acid. *P. subcapitata* Strain NIVA-CHL 1 was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany. It was pre-cultivated in freshwater medium FW04-medium (Nicklisch et al. 2008). The algae were grown in batch cultures under permanent light at 21 ± 1 °C with approximately 40 μmol photons $m^{-2}\,s^{-1}$. Purified air was pumped through the culture, and the algae were harvested during logarithmic growth. The algae were grown axenically in order to avoid any microbial contamination. The animals were fed once a day *ad libitum* indicated by suspended algae after one day of exposure.

(2) Commercial baker's yeast, *Saccharomyces cerevisiae*, Meyen ex E.C. Hansen, 1883, was kept in the refrigerator and then dissolved in small portions in *Daphnia* medium prior to feeding. This diet is considered sub-optimal (Goulden et al. 1982). This is mainly due to (i) the resistance of yeast's cell wall to digestion, so the manno-protein layer that surrounds the yeast cell can obviously not be digested by *Daphnia* and (ii) the deficiency of yeast cells in unsaturated fatty acids, both PUFA and MUFA (Coutteau et al. 1990, 1992). The animals were fed once a day *ad libitum,* indicated by small amounts of suspended yeast cells after one day of exposure.

(3) To overcome a potential deficit of spontaneous antioxidants in the second diet, the dispersed commercial baker's yeast was amended with 2 mg l^{-1} dissolved ascorbic acid, which was obtained from Sigma–Aldrich, Germany.

Humic material and exposure scenarios

In order to evaluate a potential HS-mediated lifespan modulation, the same HS preparation, HuminFeed®, was applied as with *C. elegans* (Steinberg et al. 2007), the previous series of *D. magna* (Euent et al. 2008), and the water mould (Meinelt et al. 2007). HuminFeed®[1] is a processed leonardite, which contains 43% organic carbon, has a specific UV absorption of 12.5 l $mg^{-1}\,m^{-1}$, and consists of 82% humic substances, 18% low-molecular weight compounds, and 0% polysaccharides (Meinelt et al. 2007). HuminFeed® was exposed at 0.04 and 0.9 mmol l^{-1} DOC; both concentrations are environmentally realistic, since *D. magna* is common in ponds, including even stabilization ponds of simple sewage treatment systems.

All exposures were run in 10 parallels with 5 newborn ($\leq$24 h old) female individuals each in 100 mL beakers. The animals were kept in permanent light at 21 ± 1 °C to compare the results with the previous study of Euent et al. (2008). The exposed animals were checked daily for dead individuals and their offspring were counted every second day. The exposed animals were placed into freshly prepared dissolved HuminFeed® solutions every second day and the offspring were removed. If applicable, ephippia were also counted.

Data interpretation and statistical analysis

Maximum lifespan were assessed for each condition. Alterations in lifespan values of treated versus untreated *D. magna* were specified in percentage. Statistical significance of variances of the entire lifespan was calculated by means of the log-rank test (Bioinformatics at the Walter and Eliza Hall Institute of Medical Research; http://bioinf.wehi.edu.au/software/russell/logrank/).

Mean values were calculated for reproduction; statistical significance was evaluated by one-way ANOVA (Sigma Stat 3.5, SPSS Inc., USA). Variations were calculated as standard error of the mean. Variances were considered significant at $p < 0.05$ (indicated by *) and highly significant at $p < 0.001$ (indicated by **).

Results

When fed *P. subcapitata*, exposure to HSs led to no significant lifespan modulation in *D. magna* (Fig. 1A, Table 1). There was, however, a trend in decreasing lifespan in the second part of the life of the individuals at the higher HS-concentration (Fig. 1A). In contrast to the lifespan, the mean cumulative offspring numbers per HS-exposed female were lowered in a concentration dependent manner (Fig. 1B). Maximal lifespan values are given in Table 1. No ephippia were produced with this diet.

If *D. magna* fed with baker's yeast were exposed to HSs, the lower HS-concentration tended to increase, and the higher

[1] The use of HuminFeed® is by no means an advertisement for this product. For more information of this commercial product, the reader is referred to http://www.humintech.com/001/animalfeeds/products/huminfeed.html, accessed July, 2009.

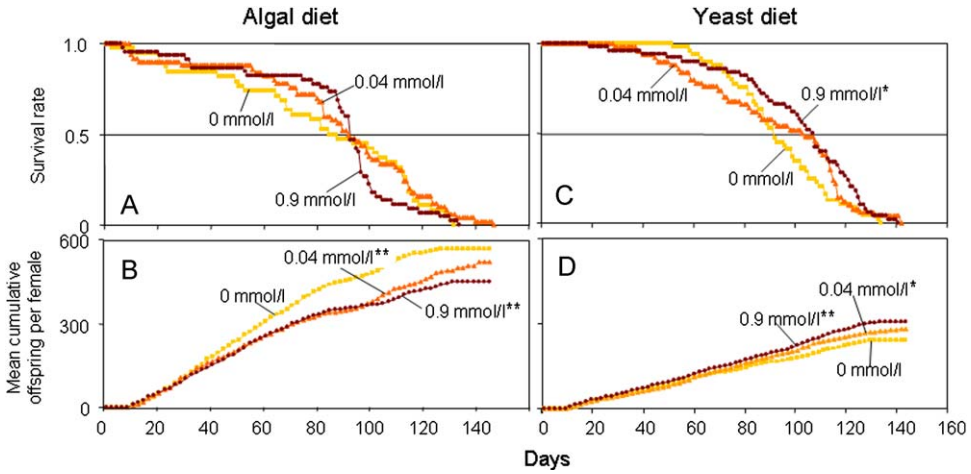

Fig. 1. Humic substance-modulated lifespan **(A, C)** and fertility **(B, D)** of *D. magna* when fed green alga, *Pseudokirchneriella subcapitata* (**A, B**) and yeast (**C, D**), respectively. $^{*}p < 0.05$; $^{**}p < 0.001$.

Table 1
Maximal lifespan and mean cumulative offspring number per *Daphnia magna* female fed different diets and exposed to dissolved HuminFeed.

Trial	Maximal lifespan			Mean cumulative offspring number per female	
	d	%	*p*	*n*	*p*
P. subcapitata control	131	100		568.84	
P. subcapitata +0.04 mmol l^{-1} DOC	146	111.45	0.483	523.35	< **0.001**
P. subcapitata +0.9 mmol l^{-1} DOC	133	101.53	0.589	450.26	< **0.001**
S. cerevisiae control	133	100		242.15	
S. cerevisiae +0.04 mmol l^{-1} DOC	141	106.02	0.239	282.66	< **0.05**
S. cerevisiae +0.9 mmol l^{-1} DOC	140	105.26	< **0.05**	308.58	< **0.001**
S. cerevisiae +ascorbic acid control	119	100		267.40	
S. cerevisiae +ascorbic acid +0.9 mmol l^{-1} DOC	142	119.33	< **0.001**	317.69	< **0.05**
The treatment *S. cerevisiae*+ascorbic acid+0.04 mmol l^{-1} DOC was terminated after four weeks, since the animals fell sick and became pink and transparent					

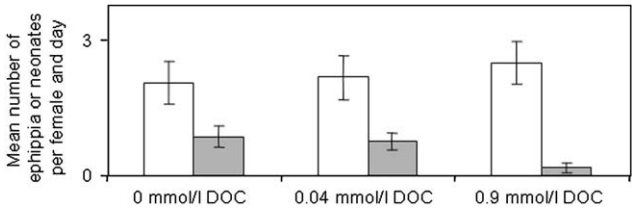

Fig. 2. Comparison of numbers of ephippia and neonates during the period of ephippia formation in *D. magna* females when fed baker's yeast, *Saccharomyces cerevisiae*, and stressed by HuminFeed. Errors are calculated as standard error of the mean. **White**: neonates; **grey**: ephippia.

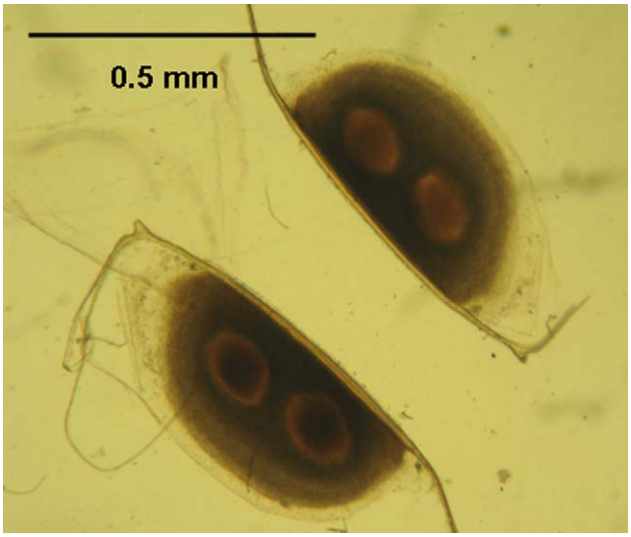

Fig. 3. Micrograph of two asexually produced ephippia with two eggs each. It is uncertain whether or not these eggs develop viable embryos.

HS-concentration significantly expanded the maximal lifespan of the individuals (Fig. 1C, Table 1), both exceeding the values of *P. subcapitata* fed control series. In addition to this, the cumulative offspring numbers were also elevated if HSs were constituents of the medium (Fig. 1D). Yet, the absolute offspring numbers only reached approximately 50% of the corresponding data of the green algae-fed animals. The body lengths of the yeast-fed females and their neonates were obviously smaller than the algae-fed ones, as evidenced by inspection during handling. Yet to minimize the handling stress of the delicate organisms, the body lengths were not determined microscopically Fig. 2.

In this treatment, *D. magna* started to produce diapausing eggs after day 15 without fertilization by males, because males never occurred throughout the entire experiment. Many of the ephippia contained two diapausing eggs (Fig. 3), so that the well understood life cycle of *D. magna* should be supplemented as sketched in Fig. 4. Without HS-exposure, approximately 3.0 ephippia per female were formed. In an almost concentration dependent manner, added HSs reduced the ephippia numbers to 2.5 at 0.9 mmol l^{-1} DOC exposure, and prolonged the period of

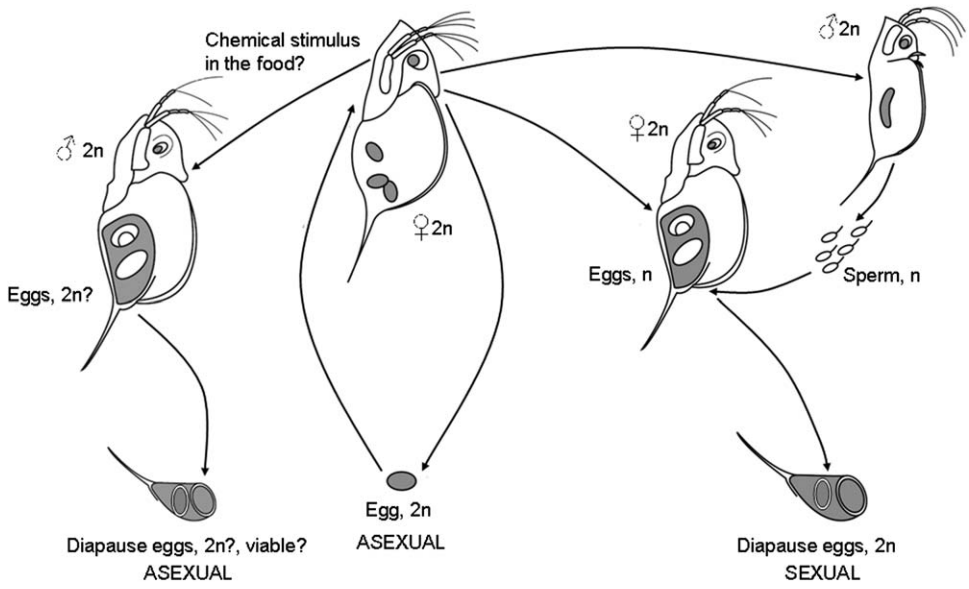

Fig. 4. Sketch of the life cycle of *Daphnia magna* in yeast-fed individuals, including the asexual production of diapausing eggs. The figure is based on Mort (1991).

their formation from 40 to 75 d (0.9 mmol l^{-1} DOC). The numbers of ephippia were low compared to the neonates released in the same period (Fig. 2).

The results with a baker's yeast diet amended with ascorbic acid did not significantly differ from the pure baker's yeast diet, and are therefore not displayed.

Discussion

In nature, food quality and quantity change temporally and force consumers into trade-offs during resource allocation for reproduction or their own maintenance (Wacker and Martin-Creuzburg 2007), whereby the latter includes both body growth and longevity (Kirkwood 1977). Very recent results from a parallel study show that poor food quality is really a biochemical stressor: baker's yeast led to reduced antioxidant capacities, which is an oxidative stress symptom (Steinberg et al. unpublished). In the present experiment, the poor food quality led to the asexual formation of diapausing eggs by an unknown mechanism. In the feeding scenarios with baker's yeast, ephippia were produced without males. Several ephippia were checked and all of them contained two eggs (Fig. 3). This finding agrees well with the statement of Hebert et al. (1989) that some genotypes produce asexual resting eggs that develop without fertilization by males. Obligate parthenogenesis has been documented in the *D. pulex* complex (in North America) and in the *D. carinata* complex (in Australia) (John K. Colbourne pers. comm.). There is only one classical report on the asexual production of diapausing eggs specifically in *D. magna* on Spitsbergen by Vollmer (1960). It is likely that a chemical stimulus induces the asexual production of diapausing eggs (Fig. 4). Although these eggs look similar to the sexually produced diapausing eggs, the molecular proof, whether or not the asexual diapausing eggs are diploid, is not yet available and is reserved to a future study.

In addition to the stress by poor food quality, dissolved HSs, being the majority of dead and living organic carbon in the water column (Thurman 1985; Wetzel 2001; Steinberg 2003), impact exposed organisms on various biochemical and molecular biological levels. The HS-mediated effects are energy consuming (Cazenave et al. 2006), which is stress by definition (Fent 2003). These results show that both stressors have a clear impact on the longevity and fecundity of *D. magna*. In algae-fed individuals, HS-mediated stress leads to reduced fecundity in *D. magna* females. This finding agrees with the previous *Daphnia* study by Euent et al. (2008), in which the fecundity was adversely affected. In contrast to the previous study, this experiment did not reveal a lifespan reduction, which is most likely due to the decreased individual density in this approach. It is interesting to note that during the present assay at 0.9 mmol l^{-1} DOC exposure, the first 50% of *Daphnia* individuals showed an increased lifespan, whereas the second 50% showed a reduced one, with the result that the whole lifespan did not statistically differ from the control (Fig. 1A).

The main outcome of both *Daphnia* series is that, under optimal food conditions, the effect of HSs on female *Daphnia* is adverse and eventually toxic: with increasing HSs concentrations the offspring numbers decrease. The underlying mechanism may be that great portions of available energy are abstracted from both catabolism and reproduction in order to activate oxygen, metabolize and/or export internalized HSs, and repair oxidative stress-mediated damages to the macromolecules. This assumption corresponds well with findings by Cazenave et al. (2006), who report on retarded developments, embryotoxicity, and lethality in HS-exposed zebrafish embryos. A report on the European mud snail, *Lymnaea stagnalis* L., also supports this assumption (Steinberg 2003). This snail was exposed to relatively low concentrations of HSs which were exotic to it. 10–20% of the individuals died when exposed to the bioavailable HS-fractions. From all these facts, it becomes obvious that internalized HSs clearly induce stress in animals.

The present study shows, however, that combined with a second stressor, such as poor food quality, HS-mediated responses do not necessarily act adversely or aggravate the adverse effects of the first stress in exposed *D. magna* individuals. For instance, when fed baker's yeast, both the lifespan of the females (Fig. 1C) and numbers of neonates per female (Fig. 1D) increased. Concomitantly, the numbers of ephippia per female decreased with increasing HS-exposure, which indicates a concentration-dependent reduction of stress strength. The situation is similar if the yeast diet is amended with dissolved ascorbic acid in the medium (not displayed). One likely explanation is that the

dissolved ascorbic acid reacted with HSs of the exposure and was therefore not internalized.

There are two potential lines of explanation for the results presented.

1. HSs may be directly utilized as a food source and partly compensate for the poor diet quality.
2. HSs directly induce longevity in exposed individuals.

HSs as a food source

When *D. magna* were fed baker's yeast, any addition of dissolved HSs increased longevity in both assays (Fig. 1C and not displayed); in the pure yeast diet, the fecundity also increased at HSs exposure (Fig. 1D). Since, by definition, dissolved HSs also include colloidal and fine-particulate organic carbon, the nutritional utilization of this carbon source has to be considered. Indeed, there is increasing evidence that this carbon fraction is directly available to filter feeding zooplankton (Gellis and Clarke 1935; Haney 1973; Salonen and Hammar 1986; Hessen et al. 1990; Jones et al. 1998). More recently, stable isotope analyses have proven that allochthonous material of terrestrial origin can contribute significantly to zooplankton biomass (Grey et al. 2001; Pace et al. 2004; Taipale et al. 2009). These papers support the idea of fine-particulate and colloidal HSs as a food source for the exposed yeast-fed *D. magna*. This line of evidence, however, can only explain parts of the finding, since it does not explain why a poor diet plus HSs expanded the lifespan of *D. magna*, while a rich one plus HSs did not. None of the aforementioned papers considers HSs to interact directly on the biochemical and molecular biological level in the animals themselves. In fact, these interactions take place and may even extend lifespan, as recently evidenced with *C. elegans* (Steinberg et al. 2007).

HSs and longevity

The results of the present study show that any HS-addition to poor diets increases the Darwinian fitness, as indicated by lifespan and offspring numbers. Nevertheless, the *Daphnia* individuals suffered from stress, which was indicated by the formation of ephippia. Signals known to induce the formation of resting eggs include food limitation, low temperatures, short photoperiod (Hobæk and Larsson 1990), chemical cues emitted by predators (Ślusarczyk 1995), and the concerted action of light and chemical cues (Ślusarczyk et al. 2005) – and one has to add: natural environmental chemicals, such as HSs.

From one major theory of aging, the *disposable soma theory* (Kirkwood 1977), it is understood that, simplified, an individual has the choice to spend a given amount of energy in its own maintenance, reproduction or longevity. The molecular basis of this aging theory is identical to the *green theory of aging* and is nicely summarized and graphed by Gems and McElwee (2003). A large set of genes has been identified whose activity is linked to longevity or aging (Murphy et al. 2003). One major gerontogene is *daf-16*, which encodes the transcription factor DAF-16, a powerful regulator of lifespan. If DAF-16 is blocked, the particular pathway applies which is triggered by the insulin-like growth factor 1 (IGF) and the adult lifespan will be reduced as shown in a variety of animals (Gems and McElwee 2003, with further references).

If the IGF pathway, however, is blocked, the organisms extend their lifespan. This has also been demonstrated experimentally with one planktonic species. Applying wortmannin, a specific inhibitor in the IGF pathway, to the rotifer, *Brachionus plicatilis* O.F. Müller, Yoshinaga et al. (2005) show that the blockage significantly prolongs the post-reproductive phase. Similar results have recently been obtained with *M. macrocopa* (Richter and Steinberg unpublished). It is understood from previous studies with HSs that it may also increase lifespan (Steinberg et al. 2007; Euent et al. 2008). One potential mechanism is the blockage of the IGF pathway by the low-molecular weight HS-compounds in analogy to wortmannin. With an optimal diet, such as the green algal food, this potential blockage by HSs is masked or overruled. Yet, with the poor yeast diets, the IGF pathway does not prevail, instead HSs have the chance to interfere within the IGF pathway and *daf-16* is no longer blocked and may, in turn, initiate the observed lifespan extensions via DAF-16.

Comparing the results within the series of the yeast diet, it becomes obvious that the lifespan extension happens in concert with, instead of at the expense of, increased numbers of offspring. Yet, even this fact agrees well with the aforementioned disposable soma theory, if it is also taken into account that both mothers and neonates are smaller-bodied than the optimally fed ones were. This means that, on yeast diets, relatively less energy is spent on maintenance than on reproduction and longevity compared to the optimal diet. This fact has been well understood for a long time (Glazier 1992).

The results presented of the simultaneous action of two different stressors show that two potential adverse stressors do not necessarily mutually amplify each other and may open new perspectives to zooplankton ecology. It is obvious that stressors, such as low food quality, even act, in concert with the biogeochemical matrix, beneficially on the Darwinian fitness of individuals with as yet not understood feedback on higher levels, such as populations and communities.

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